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Preferential Selection of Central Pathways by Regenerating Optic Fibers

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The time-course and general features of optic nerve regeneration in goldfish were followed in sections prepared at spaced intervals between 3 and 67 days after nerve section. Differential route and destination preferences of fibers from different parts of the retina were then tested by removing specific portions of the retina in combination with complete section of the optic nerve. With the dorsal half of the retina destroyed, surviving ventral fibers became segregated beyond the nerve scar and selectively entered the medial tract to connect with dorsal tectum. Conversely, when dorsal retina remained intact, the regenerating fibers filled selectively the lateral tract and the ventral tectum. Fibers from the posterior (temporal) hemiretina invaded the anterior portion of the tectum and did not extend into the posterior regions. Conversely, those from the anterior hemiretina bypassed the anterior zones to innervate the posterior tectum. Fibers from the center of the retina, after reaching the parallel layer within the tectum, bypassed the plexiform layer in the margin to connect only in the central zone. The plexiform layer in the marginal zones was innervated only when fibers were available from the peripheral retina. The results furnish direct microscopical evidence for the orderly selective termination of optic fibers in the brain centers. They also demonstrate a remarkable and unexpected (presumably chemotactic) selectivity in the tendency of different retinal fiber groups to choose and to follow specific central pathways en route to their synaptic destinations. The thesis that specific chemical affinities govern the formation and maintenance of neuronal associations is extended on the basis of the present results to include the patterning of central fiber pathways.

Introduction

Regeneration of the severed optic nerve in fishes and amphibians leads, under optimal conditions, to good recovery of visual function (21).

[1] This investigation was supported in part by the Frank P. Hixon Fund and by a PHS research grant (M3372) from the National Institute of Mental Health, Public Health Service. A brief presentation of the results was made at the 1960 Fall meetings of the American Physiological Society (3).

The perception of color, directionality, movement and pattern as well as visual acuity have all been shown to be recovered at a high level approximating that of normal vision. Further, visual discrimination habits involving color, brightness and pattern, learned by fishes prior to section of the optic nerve are reinstated by the regeneration process and in cichlids, *Astronotus ocellatus*; these have been found to exhibit interocular transfer and to survive the combined ablation of forebrain plus cerebellum (1, 2).

The collected evidence including histological observations and mapping data obtained with localized tectal lesions (16-21) was taken to indicate that the regenerating fibers re-establish their central connections on an orderly plan that systematically reduplicates the original topographic projection of the retina on the tectum in accordance with the projection pattern characteristic of the species. It has been suggested that the establishment of this topographic projection may be regulated by specific chemical affinities between matching loci in retinal and tectal fields respectively, the affinities being established by embryonic differentiation gradients that sweep over retina and tectum early in development, first, along the rostrocaudal axis and then the dorsoventral axis.

Extension of the experiments into early embryonic stages has brought more direct evidence for the presence of at least the two main retinal gradients and for the respective timing of their establishment (22, 23). Recent mapping of retinally evoked electrical potentials in the tectum of anurans following optic nerve regeneration by Gaze (4, 5) and by Lettvin, Maturana and associates (12, 13) has brought further support for the inference that the regenerating optic axons achieve selective reconnection with their original loci of termination.

A question long at issue in the foregoing is whether the experimental data including the electrophysiological maps might not be accounted for without assuming any orderly regeneration of central connections. It has been pointed out that certain coding-decoding schemes might in theory account for all the observed behavioral data without the assumption of anything more than a randomized reconnection (11). The available data could also be accounted for in terms of the resonance principle of Weiss (24), which was designed expressly to explain organized function within randomized networks, and in networks deranged by surgery and mis-regeneration.

Neither the lesion nor the electrical mapping studies have been decisive on this question, in part because of uncertainty as to whether

the observed scotomata and the tectal potentials reflect presynaptic or postsynaptic effects. Postsynaptic activity could yield the orderly maps obtained after a purely haphazard regrowth if something caused the resultant synapses to be functionally effective only within the appropriate loci. This might be the case, for example, in the above resonance or coding schemes. The exact source of the tectal potentials is considered uncertain by Gaze (4-6), while Maturana and his colleagues (14) favored the terminal arborizations of the optic axons as the probable origin. In the latter case the possibility of random meandering and widespread arborization is not excluded. The electrical studies of Gaze (6) and Jacobsen (8) have suggested the possibility of a scheduled timing of fiber ingrowth and the presence of an early diffuse nonlocalized phase of regeneration that subsequently may or may not be transformed into a systematized topographic organization. They have also pointed out another complicating factor in the presence of a previously unrecognized projection of each retina to the ipsilateral as well as to the contralateral tectum (7). The overlapping ipsilateral projection is not in register with the main contralateral projection.

The following was undertaken in the hope that some of the above and other uncertainties about the regeneration process might be clarified. The experiments, started in 1958, were undertaken on the supposition that the sectioned optic fibers probably remain fortuitously scrambled in regeneration until they regain the plexiform layer of the tectum, an inference drawn from earlier results on the frog (21). It thus proved something of a surprise to find evidence of highly discriminative, presumably chemotactic, differences among the regenerating fiber groups enabling them to select and to follow preferentially their own original pathways en route to their specific terminal stations.

Materials and Procedure

Most of the experiments were carried out on goldfish, *Carassia aurata*, about 5 to 9 cm in standard length. Selected aspects of the work as mentioned in context below were repeated in the cichlid, *Astronotus ocellatus*, in which the visual system is more highly developed. Results in the two species were essentially the same; the following text applies principally to the goldfish unless otherwise indicated.

Normal Anatomy with Reference to the Regeneration Problem. The main retinal projection to the optic tectum in the goldfish is diagrammed in Fig. 1. The optic nerves cross completely at the chiasm so that each

eye connects to the contralateral optic lobe. Before entering the midbrain tectum the primary optic tract divides into two main bundles, the medial and lateral optic tracts. These course along the medial and lateral circumference of the optic lobe, respectively, giving off fibers all along the periphery which they turn radially into the optic tectum where they run in a superficial parallel layer to reach their local synaptic zones.

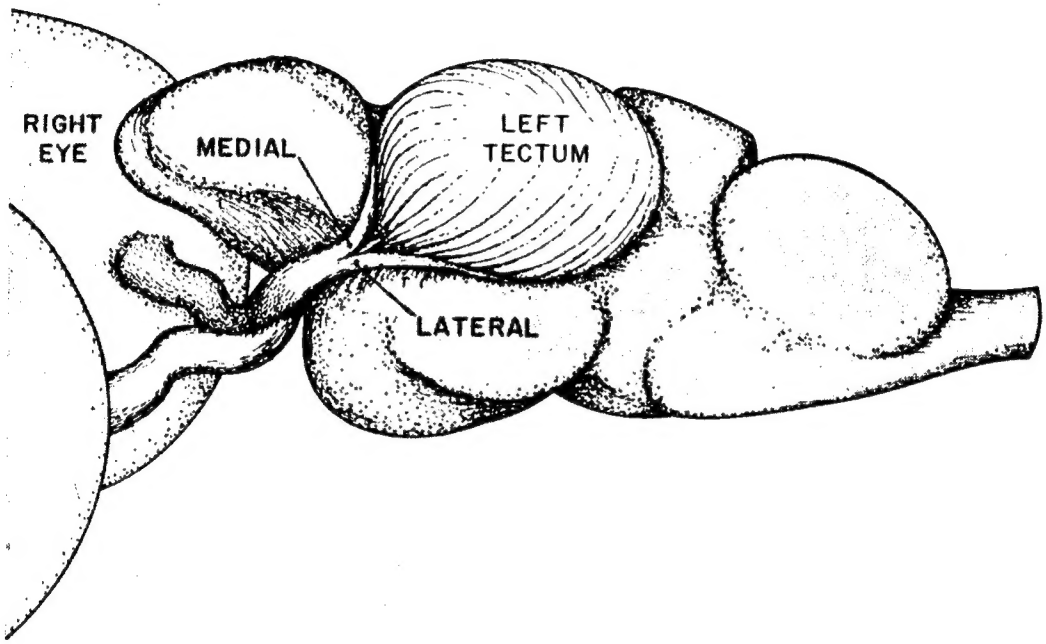

FIG. 1. Schematic drawing of goldfish optic system showing division of main optic tract into medial and lateral bundles and their relations with midbrain tectum.

The medial tract, as shown below, consists of fibers arising in the ventral part of the retina. The fibers spread fanlike into the dorsal portion of the tectum from its medial border. Conversely, the fibers of the lateral tract arise in the dorsal retina and spread from the ventral border of the lobe into its ventral and lateral areas.

Along the circumference of the tectum small bundles of fibers separate from the main medial and lateral tracts to enter the tectum where they then proceed in a relatively straight, roughly parallel course toward the center of the optic lobe. Fibers originating in the posterior (temporal) quadrants of the retina are destined for the anterior or rostral quadrants of the optic lobe and accordingly they leave the main tracts earlier, whereas those from the anterior (nasal) retina remain in the tracts until they reach the posterior regions of the tectum.

After the fibers have run their course in the superficial parallel layer of the tectum they exit from this layer by dipping centrally into an underlying plexiform layer. In accordance with the principle of topographic correspondence between retina and tectum, fibers arising from the outer periphery of the retina exit from parallel to plexiform layers abruptly after entering the tectum. Whereas those from more and more central points along a given retinal radius successively delay their entrance into the plexiform layer until reaching correspondingly more central zones of the tectum.

Thus the normal central course of the optic fibers is so laid out that, in effect each regenerating optic axon finds itself confronted by a kind of multiple Y-maze. In order to return to the tectum by its own original pathway, a given regenerating axon must first select correctly the medial or the lateral tract. After that comes a continuous series of decision points along the circumference of the tectum at each of which the fiber can either turn radially into the parallel layer of the tectum or continue to push ahead tangentially in the main tract. Upon entering the parallel layer of the tectum, each fiber again meets a series of choice points as it advances from periphery to center wherein it can either dip downward into the plexiform layer or continue to grow on farther centrally within the parallel layer.

Finally, after entering the plexiform layer the different types of retinal fibers must again select presumably the proper tectal units for appropriate patterns of synapsis, not only with reference to tectal topography and directionality in vision but also with respect to other dimensions of physiological specificity. For example, fibers for red, blue, green, and yellow, those for luminous flux, the "on", "on-off" and "off" fibers, and perhaps other specialized detector types (11–14), must each form its own characteristic pattern of tectal linkages after arrival at the correct locus of the tectal map. Within the plexiform layer it can be seen that the fibers begin to bifurcate and the branches get thinner than the parent fiber, lose their myelin, and no longer run parellel, but course erratically in all directions, eventually to synapse with tectal neurons.

Experimental Plan. In a first group of thirty-nine goldfish one optic nerve was severed in order to study the time-course of optic fiber regeneration in sections prepared at spaced intervals varying between 3 and 67 days after nerve section. In cases with fully regenerated nerves, the fiber pattern within the nerve, tracts, and in the parallel and plexus layers of the tectum were compared with those of normal fish.

In a second group of seventy-four fishes, removal of a large sector of one retina was combined with section of the optic nerve of the same side in order to determine the course taken by the fibers regenerating from the remaining intact retinal areas, as follows:

(a) Ventral-dorsal series. The ventral (or, in other cases, the dorsal) half of one retina was removed and the optic nerve of the same side completely severed. As a control in some cases, the opposite nerve was sectioned as well.

(b) Anterior-posterior series. The anterior (or the posterior) half of one retina was removed and the optic nerve of the same or both sides completely severed.

(c) Central-peripheral series. A partial or complete peripheral ring of retina was removed and one or both optic nerves severed.

Many of the operated fish yielded little or no information owing to inadequate differentiation of the stain, poor placement or excessive extension of the retinal lesion, inadequate regeneration, poor plane of section, and other factors encountered in the early exploratory procedures. Further cases were prepared in each series until a convincing mass of evidence was obtained. The described results represent a composite impression drawn from the total but based largely on the several best cases in each series. In most cases the histology was checked at 17 to 25 days after nerve section, though a few were taken earlier and later for special purpose.

Surgical Procedure. The surgery was performed out of water under a stereoscopic microscope. The fish were anesthetized in a 0.5% solution of tricaine methanesulphonate (MS 222 Sandoz Chemical Co.) in conditioned tap water. The anesthetized fish was wrapped in a wet cloth and placed in a plasticene mold to hold it in the desired position. Anesthesia was maintained during surgery by dropping a dilute solution of tricaine over the gills.

The nerve section was performed within the orbit. The approach was posterodorsal with the eyeball tilted anteroventrally. The nerve was severed fairly close to the eyeball at about one-fourth of its length to the chiasma. The nerve was cut and broken with finely pointed forceps leaving intact only a small strand of the outer dural sheath. This remnant of the outer sheath was left to hold the nerve stumps close together and to serve as a bridge for the regenerating fibers.

The retinal lesions were made in different ways. In the earlier cases the retina was approached through the pupil after cutting the cornea

and lifting the lens. The area of retina to be removed was pinched and teased with fine forceps after which the fragments were delicately sucked away. The lens was then replaced and the shreds of the cornea rejoined.

In the majority of cases, the retina was approached by making a cut along more than a half of the border of the anterior face of the eyeball with fine scissors. The front part of the eyeball with the attached lens was then lifted and the whole retina clearly exposed. In a few of these the portion of retina to be removed was cut away together with the external layers of the eyeball. In most, however, an incision was made through only the retinal layer with the sharpened edge of a fine wire loop, thus separating the selected part of the retina from the adjacent portions without injury to the outer membranes. Care was taken to avoid damage to the peripapillar area. The isolated sector of retina was then removed with fine forceps. In practice this latter became the preferred method and most of the results described below were obtained with this technique.

Histology. At sacrifice the retinal lesions were checked. Under deep anesthesia, the eyeball was opened and the retina examined in the living state with the stereomicroscope.[2] Afterward the cranium was opened and the whole head quickly immersed in abundant fixative (18 parts of 80% ethanol, 1 part acetic acid, 1 part formalin). A few hours later, the brain with the optic nerves and eyes attached was carefully dissected free and placed for 24 to 48 hours in fresh fixative. The fixed brains were then embedded in paraffin, serially sectioned at 15μ, and the sections stained with the Bodian protargol method. In most cases, the normal fibers stained black with this method while the newly regenerated fibers acquired an intense pink or reddish hue. The contrast seemed to be enhanced by increasing the prescribed amount of protargol until the pH approached 8.8 and by reducing the amount of copper to about 0.25 gr.

Observations

Time-course and General Features of Optic Nerve Regeneration. The speed of the regeneration process, as judged from the time required for recovery of visual function (14 to 18 days in *Carassius*; 25 to 35 days in *Astronotus*) was found to vary in relation to various factors such as

[2] It is of incidental interest that extensive regeneration of the ablated retinal areas was found to be well underway in many cases. Further details of this retinal regeneration are left for another study.

the size and age of the fish, the season, and the degree of hemorrhage and displacement of the cut stumps. The following times indicate a rough summer-season average for goldfish 5 to 9 cm long.

At 3 to 4 days after nerve section, the whole distal segment of the optic nerve and the two bundles, medial and lateral, into which the optic nerve divides before entering the tectum, appear to be degenerated. Within the tectum, the two main bundles are completely degenerated up to the point at which the fibers spread into the parallel optic layer. Within the retinic layers of the tectum, however, signs of fiber degeneration are not yet noticeable at this time. It is known that the process of elimination of the retinic fibers of the cortex is a relatively slow one (10). In the proximal segment of the severed optic nerve also there were signs in many cases of some retrograde degeneration due perhaps to surgical trauma, such as stretching of the nerve and of the retinal artery which were difficult to avoid.

At 4 to 7 days after section, the optic fibers have started to regenerate and have become mixed and entangled in a dense and swollen neuromatous growth between the two nerve stumps. Here one cannot recognize any orderly pattern of regeneration, although the possibility that the fibers tend to segregate cannot be ruled out. As soon as the entangled regenerated fibers have reached the central stump of the severed nerve, they take again a grossly parallel alignment. Where the fibers emerge from the scar one can observe a progressive rearrangement; the fibers gather in groups becoming more and more conspicuous. In most cases, when the regenerated nerve reaches the point of separation into medial and lateral bundles the fibers destined for one or the other tract seem to have gathered in advance toward the corresponding lateral or medial side. Sometimes one sees small bundles of fibers transferring from one nerve sector to another just before the point of bifurcation.

At 10 to 12 days after nerve section the regenerated fibers start to reinnervate the optic lobe. Small groups of fibers exit from each bundle at different points along the circumference of the lobe and enter the tectum to course in the superficial parallel layer. This layer is markedly thicker in the proximity of the bundles, along the inner and ventral borders of the optic lobe, and becomes progressively thinner toward the central areas of the cortex. Already fibers may be seen leaving the parallel layer to enter the underlying plexiform layer particularly in the border regions.

At 14 to 18 days after nerve section, when visual function is being re-

instated, the plexiform layer formed by the regenerated fibers is visible in all areas of the optic tectum. The layer as a whole is markedly thicker and the fiber bundles are more richly interwoven than in the normal tectum as can be seen in Fig 2. Also the distinction between parallel and plexiform layers is less clear.

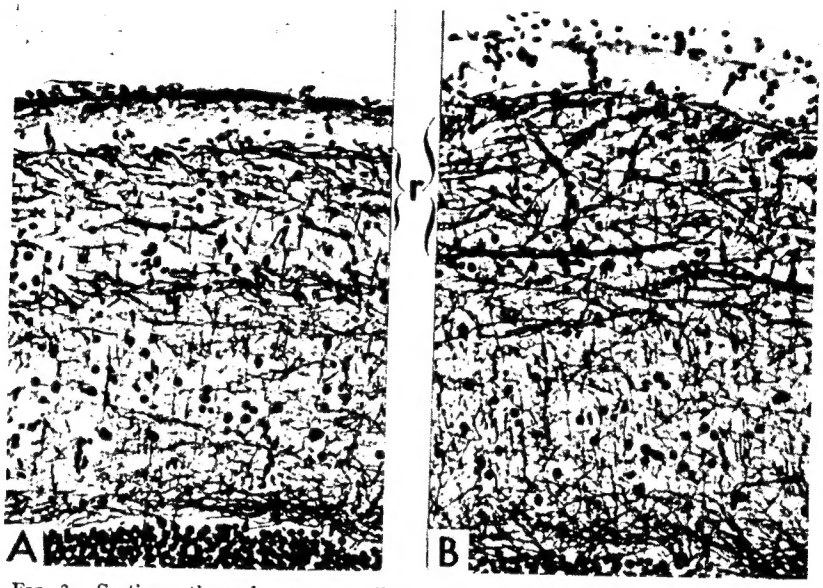

FIG. 2. Sections through corresponding parts of normal (A) and reinnervated (B) tectum of goldfish 16 days after section of one optic nerve. Layers with retinal fibers are indicated at r. Bodian stain; 160 ×.

The regenerated fibers were clearly distinguishable from the normal ones for several weeks after recovery of vision, not only by the reddish hue that they assumed with the modified Bodian stain, but also by their structure and their interrelationships. The normal optic fibers, which are myelinated in the nerve and in the parallel layer of the cortex and unmyelinated in the plexus layer, appear when stained by the above method, as black, well-individualized filaments. The regenerated fibers, on the other hand, which are not yet myelinated when vision returns, are packed together in the nerve in small bundles within which single fibers are not clearly distinguishable. In the optic layers of the cortex the bundles of regenerated fibers appear thicker, often ribbon-shaped, and not well individualized (Fig. 2).

During the following weeks the regenerated fibers undergo a slow process of maturation toward the normal aspect. They become better individualized as the myelin sheath is forming, assuming then a filamentous, normal appearance. This process does not take place simultaneously in the whole fiber, but proceeds from the nerve cell in the retina toward the terminal arborization.

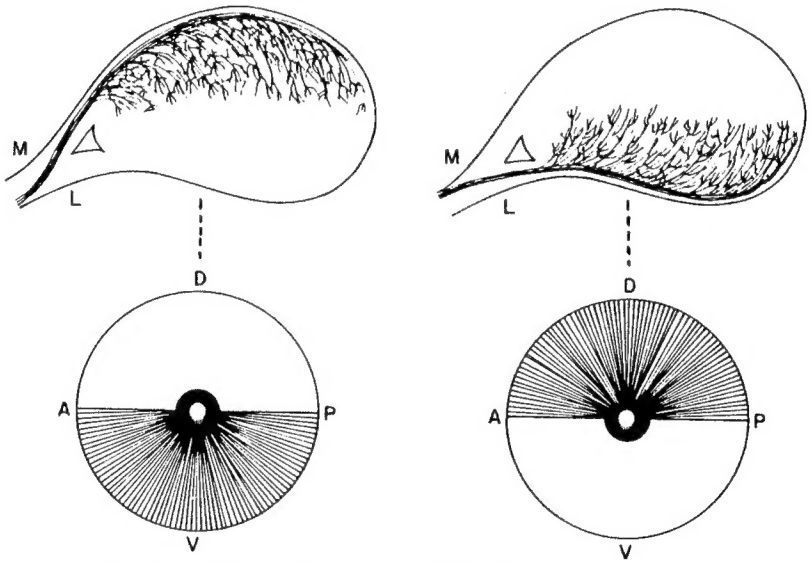

FIG. 3. Schematic representation of the regeneration fiber patterns obtained with nerve section and ablation of dorsal or ventral hemiretina, respectively.

Selectivity of Regeneration. Ventral-dorsal series. The first set of experiments was aimed at determining whether the fibers regenerating from the ventral or dorsal half of the retina would show any preference to enter the medial or, respectively, the lateral optic tract. The results for this series are schematically summarized in Fig. 3. When the dorsal half of the retina was removed and the optic nerve of the same side severed, the remaining fibers originating in the ventral half of the retina regenerated and were found to enter the medial bundle. The route of the lateral bundle was left empty with the exception of a few fibers that presumably originated mainly in the zone immediately adjacent to the papilla in the dorsal retina, which was intentionally left intact during the

surgery. The retinic parallel and plexiform layers were restored in the dorsal tectum only. Conversely, when the ventral half of the retina was

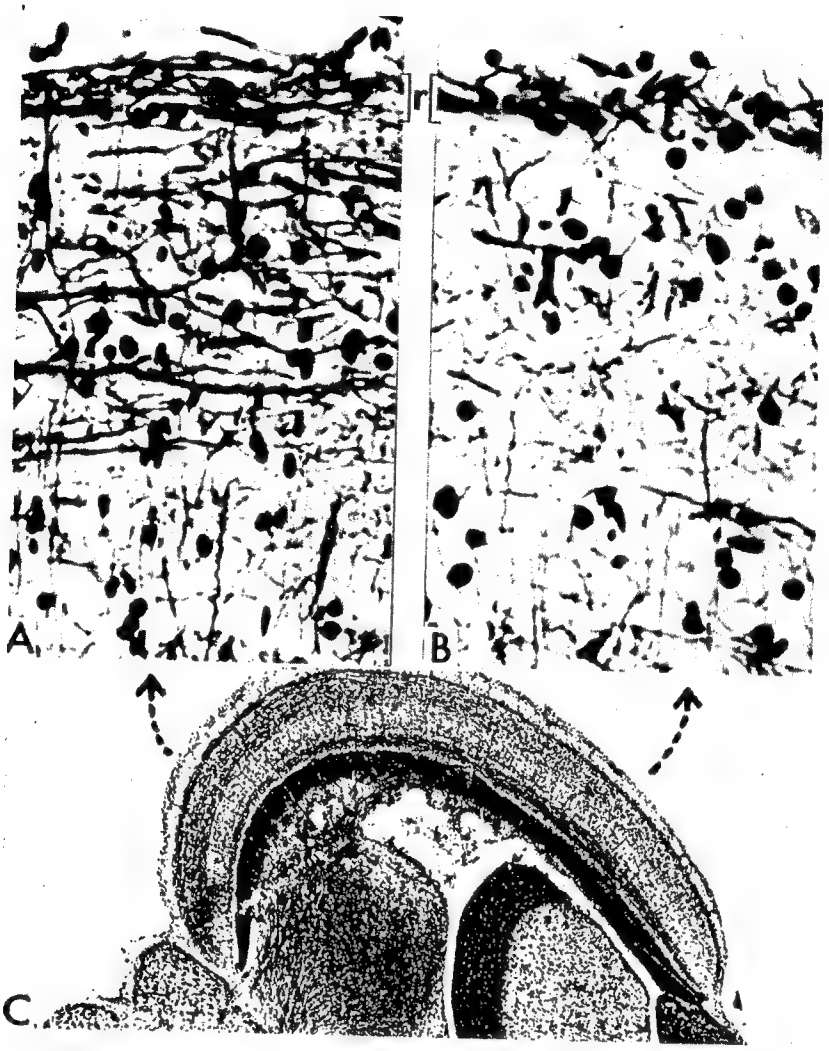

FIG. 4. Cross section of goldfish optic lobe (C) following optic nerve regeneration with ventral hemiretina ablated; details from the reinnervated ventral zone (A) and from the dorsal zone lacking regenerated fibers (B). The level of the parallel retinic layer is indicated at r. Bodian stain; A and B, 360 ×.

removed, nearly all of the regenerated fibers were found to enter the lateral bundle, and only the ventral half of the cortex was reinnervated.

In each case the one half of the tectum showed an abundant supply of regenerated fibers both in the parallel and plexus layers, whereas the other half appeared generally void of regenerated fibers (Fig. 4). The border between the empty and reinnervated halves of the cortex was not abrupt but was fairly distinct, especially in *Astronotus*. More exact

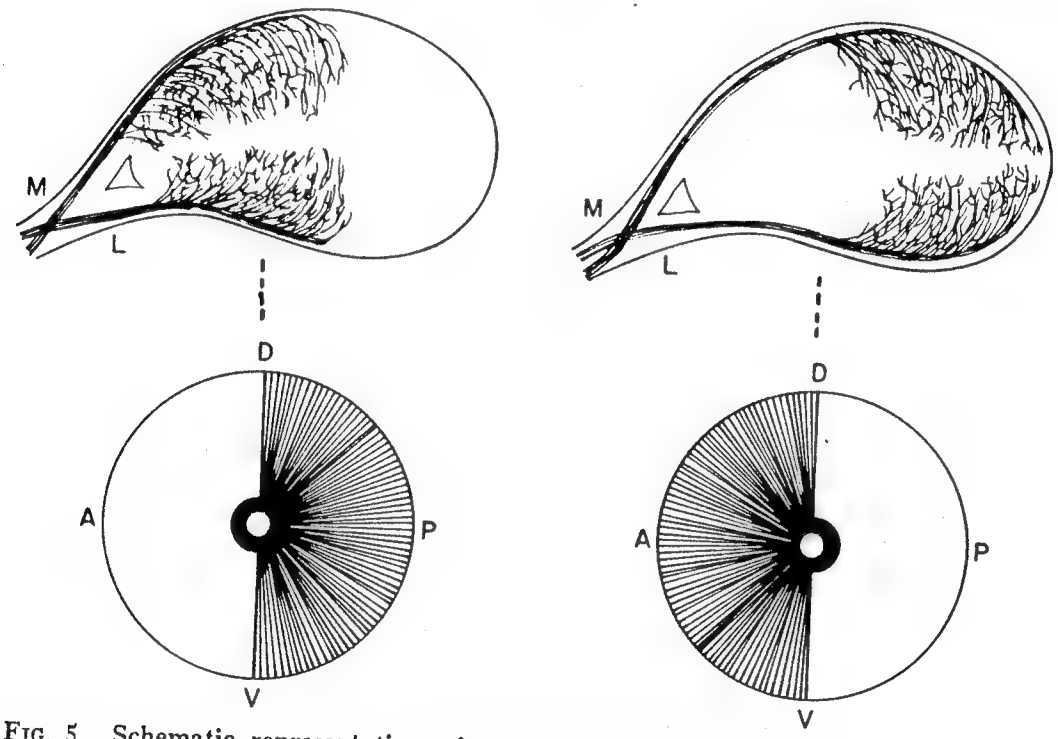

FIG. 5. Schematic representation of regeneration patterns obtained with removal of anterior (nasal) or posterior (temporal) hemiretina, respectively.

determinations along this line must be left for further study with different procedures.

In some cases the two types of retinal lesion were made in the same animal, i.e., a ventral removal was made in one eye and a dorsal removal in the other and both optic nerves sectioned. Under these conditions the double reverse effects were evident and easily compared in the same transverse sections.

Anterior-posterior series. The second set of experiments was designed to test for preference on the part of the regenerating fibers for the point of entrance into the tectum along its circumference. The results are schematically summarized in Fig. 5.

When the posterior half of the retina was removed and the nerve of the same side severed, the regenerating fibers arising from both ventral and dorsal quadrants of the anterior retina split into two groups, one of which entered the medial and the other the lateral bundle. Within both

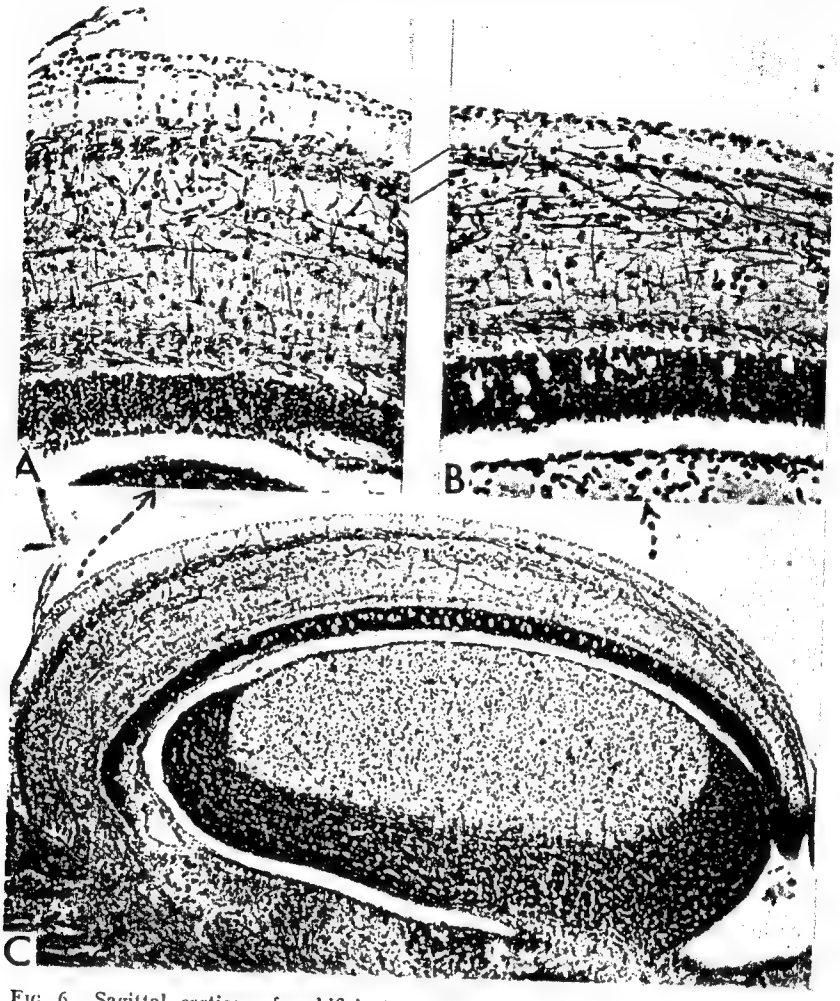

FIG. 6. Sagittal section of goldfish tectum (C) showing selective reinnervation of posterior zone following ablation of temporal hemiretina. Enlarged details from anterior (A) and posterior (B) sectors show plexiform layer to be absent in anterior region but richly developed posteriorly. Bodian stain; A and B, 170 ×.

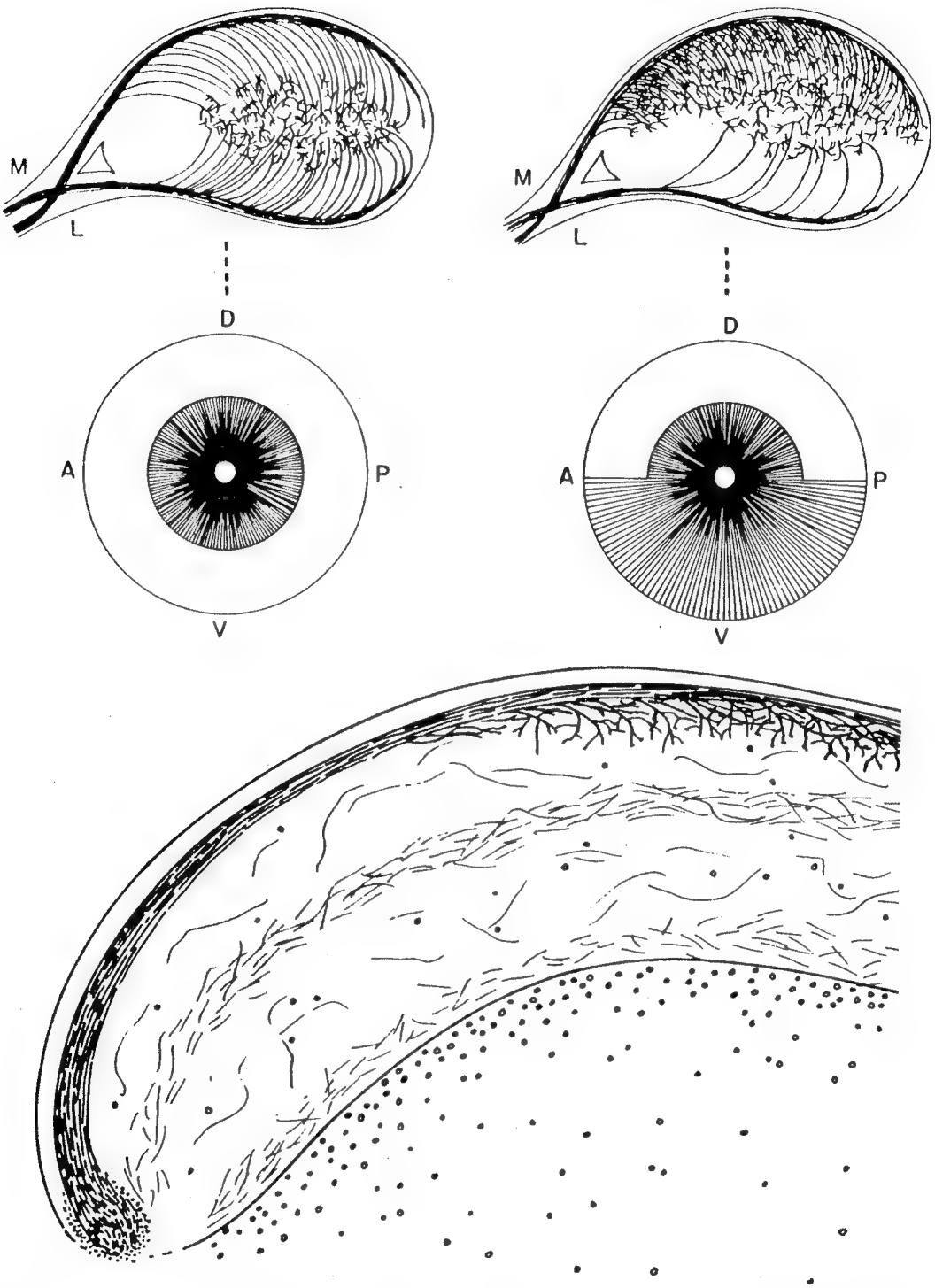

FIG. 7. Type of regeneration patterns obtained with removal of peripheral retina. Below: Enlarged detail of the result as viewed in a transverse section of tectum.

tracts the bulk of the fibers were found to remain in the bundle until they approached the posterior regions of the tectum. A few fibers were evident anteriorly in the parallel layer but they passed through without entering the plexus layer. The plexiform layer was formed only in the posterior of the tectum (Fig. 6).

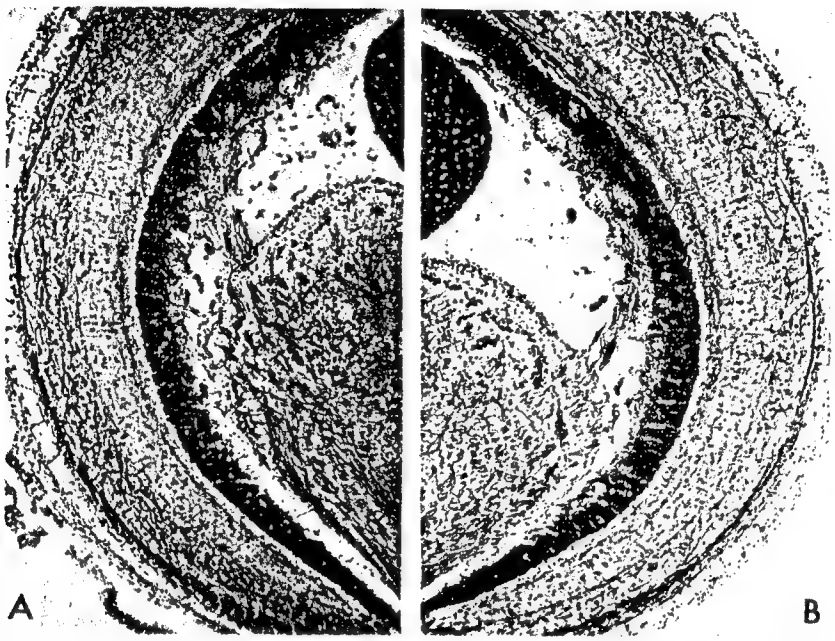

FIG. 8. Cross sections of ventral region of reinnervated right and left optic lobes. A, complete reinnervation with no retinal lesion; B, selective by-passing of plexiform layer in marginal zone (lower half of photo) after removal of dorsal periphery of retina. Bodian stain; 68 ×.

In the other fishes in which the anterior portion of the retina was excised and the nerve severed, the fibers similarly split into two groups, one of which entered the medial and the other the lateral tract. However, in these the fibers from both tracts entered and reinnervated the anterior regions of the tectum and did not extend back into the posterior regions in either the parallel or plexiform layers.

Radial series. The third set of experiments was designed to determine whether the regenerating fibers after their entry into the parallel layer of

the cortex, would show any preference in entering the underlying plexiform layer. The results are schematically summarized in Fig. 7.

In this series the peripheral retina was first removed from just the dorsal half and the same or both optic nerves were severed. In these fishes the regenerating fibers coming from the intact ventral retina entered the medial bundle and reached their terminations throughout the dorsal part of the tectum. The fibers coming from the central peripapillar portion of the dorsal retina, on the other hand, after running through the lateral bundle, entered the parellel layer of the cortex but remained in this layer until they arrived at the central zone of the tectum. Thus the marginal part of the ventral tectum contained a parallel layer but the plexiform formation was absent (Fig. 8).

When a complete peripheral ring of retina was removed, leaving intact only the center of the retina, the regenerating fibers entered the tectum through both the medial and lateral bundles, but in neither case after entering the tectum did the fibers descend into the plexiform layer until they had reached the central zone. The entire margin of the optic lobe was thus by-passed by these fibers arising from the central retina.

Discussion

The foregoing provides direct microscopical confirmation of earlier deductions that the regenerating optic axons reconnect selectively in matching loci of the tectal field to restore an orderly topographic projection of the retina upon the tectum. In addition the findings disclose for the first time an unexpectedly high degree of specificity in the choice of central pathways taken by the fibers en route to their terminal stations. The results would appear to dispell any remaining doubts that the growing optic fibers are destination-bound. They appear to be not only rather specifically destination-bound, but also definitely inclined to follow particular routes to their respective destinations.

It would seem most probable that the selective growth along specific central routes by the different fiber types is chemotactic in nature and is based on biochemical specificities, presumably similar to or identical with those we have inferred to be operative in the formation and maintenance of functional synapses. The same set of anteroposterior and dorsoventral gradients in retina and tectum needed to explain the topographic reconnection pattern would be sufficient to account also for the orderly tract and radiation formations observed.

In addition it would seem likely that the various extraneuronal

elements in and immediately around the nerve routes, must also possess distinctive chemical properties. For example, the opposite walls of the main nerve trunk just before its bifurcation into medial and lateral tracts may differ sufficiently to favor an advance segregation of fibers into medial and lateral bundles. We may imagine any growing fiber tip to be continuously testing its surroundings as it advances, by extending and retracting a number of microfilaments along the surrounding cellular surfaces in all directions. Most of these scouting filaments are then withdrawn in favor of the one that finds the substrate most favorable and the extension of which also is most in accord with the intrinsic individual growth properties of the given fiber.

Our thesis that specific chemical affinities govern neuronal synapsis (1–3, 15–21) may be extended on the basis of the present findings to include the patterning of central fiber pathways. Not only the details of synaptic association within terminal centers, but also the routes by which the fibers reach their synaptic zones would seem to be subject to regulation during growth by differential chemical affinities. This becomes essentially a chemotactic interpretation and takes us a long way from the prevailing doctrine of the 1920's, and 30's which held that nerve fiber outgrowth and termination is basically nonselective, with chemotropic and electrical factors seemingly ruled out in favor of mechanical contact guidance. The well-demonstrated importance of mechanical factors (25) is not to be minimized, but the present evidence suggests a reconsideration of some of the more ancient ideas of chemotaxis, chemotropism, neurotropism and of chemical selectivity in general in the guidance of nerve fiber growth and connection particularly within the central nervous system.

We found no indication in these fishes of an early nonselective phase of regeneration. Selectivity was already evident with respect to choice of medial and lateral tracts and the invasion of the tectum as early as 12 days after nerve section. The possibility of initial diffuse and excessive growth of fibers with subsequent elimination of the more devious and circuitous branches remains to be considered; however, with reference to the local terminal growth on microscopical dimensions within the plexiform layer.

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